

# **OF MICE AND MEN: TRANSLATIONAL STUDIES OF POSTTRAUMATIC STRESS DISORDER**

by

Ashlee Brooke Filippone

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This thesis was presented

by

Ashlee Brooke Filippone

It was defended on

April 1, 2010

and approved by

Anne Germain, Ph.D, Department of Psychiatry, University of Pittsburgh

Christopher O' Donnell, PhD, Department of Medicine, University of Pittsburgh

Michael Sayette, PhD, Department of Psychology, University of Pittsburgh

Todd Arnedt, PhD, Department of Psychiatry, University of Michigan

Thesis Advisor: Anne Germain, Ph.D, Department of Psychiatry, University of Pittsburgh

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Ashlee Brooke Filippone, B. Phil

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Sleep disturbances are the most commonly reported symptom associated with posttraumatic stress disorder. Experiencing sleep disturbances such as nightmares and insomnia early on after trauma exposure has shown to significantly increase the risk for developing the daytime symptoms (i.e. reexperiencing and hyperarousal) of PTSD. Sleep disturbances (nighttime symptoms) are often resistant to current first-line treatments used to alleviate PTSD's daytime symptoms. But why have sleep disturbances become such a defining characteristic of PTSD? Recent research suggests that the role sleep plays in this trauma-induced anxiety disorder is not only a diagnostic symptom, but may actually be a causal factor for the onset and persistence of PTSD's daytime symptoms. However, it is difficult to study the role of sleep disturbances in the development and maintenance of PTSD due to human limitations after trauma. Alternatively, animal models that use fear conditioning to elicit a fear response similar to what we see in PTSD in humans may yield further insight to understanding the role of sleep in trauma responses and guide the development of more effective treatments for PTSD. Here, we propose a novel animal model of conditioned fear to study the impact of trauma on insomnia and nightmares. A two-part fear conditioning paradigm was designed to validate use of a mild transient hypercapnia (mTH) as a novel conditioned stimulus for fear conditioning. Fear responses were measured by both behavioral and physiological changes. In addition to measures of sleep- wake states, physiological measurements of heart rate, blood pressure and EMG recordings were continuously monitored during both wakefulness and sleep in mice. We expected to see physiological changes indicative of fear responses in addition to changes in sleep architecture

(specifically in NREM and REM sleep) commonly seen in fear conditioning. The goal of this two part study is to validate the use of (mTH) as an effective condition stimulus for fear conditioning. Validation of this model will allow for future presentation of mTH during sleep without awakening the mice in a new paradigm that can probe sleep.

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## **1.0 INTRODUCTION**

### **1.1 WHAT IS POSTTRAUMATIC STRESS DISORDER (PTSD)?**

Posttraumatic stress disorder (PTSD) is a complex anxiety disorder resulting from trauma exposure. More specifically, the *Diagnostic and Statistical Manual of Mental Disorders IV- TR*, defines PTSD as the development of symptoms following the exposure to an extreme traumatic stressor that elicits a response of fear, helplessness and horror (American Psychiatric Association, 2000). These symptoms must be present for at least one month after the traumatic event has occurred, and include re-experiencing symptoms (such as nightmares, intrusive thoughts and images), hyperarousal symptoms (such as insomnia and exaggerated startle reactions), and avoidance (such as efforts to avoid thoughts and reminders of the trauma), all causing significant distress and functional impairment. PTSD is the only psychiatric disorder that specifies two distinct types of sleep disturbances, with insomnia and nightmares as core features of this pervasive disorder. Thus, PTSD can be defined as having both daytime and nighttime symptoms. PTSD is highly prevalent, with a lifetime prevalence rate in the general population of anywhere from 1- 10 percent and anywhere from 15- 30 percent of current military personnel meet the diagnostic criteria for PTSD (Breslau, et al., 1998; Weiss et al., 1992).

Currently, first-line treatments of PTSD include antidepressants and cognitive-behavioral approaches (Forbes, et al., 2009; Stein, Imler, & McAnda, 2009). However, these have limited

benefits on sleep symptoms of PTSD. Rather, pharmacological sleep treatments (such as the  $\alpha$ -1 antagonist drug Prazosin), cognitive behavioral therapy (CBT) for insomnia, and image rehearsal therapy (IRT) for the treatment of nightmares are all used as sleep-focused treatments for PTSD (see Lancee et al., 2008 for review).

### **1.1.1 Sleep Disturbances in PTSD**

Sleep disturbances are one of the most commonly reported symptoms among combat exposed veterans with PTSD, where up to 90% of patients report sleep disturbances (Jukie et al., 1999) (Lancee, Spoormaker, Krakow, and Bout, 2008; Neylan et al., 1998). Among these sleep disturbances, nightmares and insomnia have the highest prevalence rates of 52 percent and 90 percent respectively (Neylan et al., 1998; Green, 2003; Jukie et al., 1999). According to the American Psychiatric Association (*DSM-IV- TR*), insomnia, is defined as the difficulty someone has of falling sleep or maintaining their sleep state in relation to non-restorative sleep (2000). Nightmares, within the context of PTSD are defined as extremely frightening, recurrent dreams related to the trauma that cause distress and result in the person waking from sleep (American Psychiatric Association, 1994). Studies have shown that 50- 70% of PTSD patients report having trauma related nightmares.

Extensive research has been done on the effects PTSD has on both NREM and REM sleep. In a literature review by Lamarche & Koninck (2007), it is suggested that “anxiety dreams” whether nightmares (which occur during REM sleep) or night terrors (occurring in NREM sleep and are accompanied by body movements) are disruptive to the sleep quality of PTSD patients. Meta- analyses on post- trauma NREM and REM sleep have shown an increase in the amount of NREM sleep, accounted for by an increase in stage 2 (light) sleep and a

decreased in slow wave sleep; evidence of disrupted, non- restorative sleep (Kobayashi, Boarts, & Delahanty, 2007). Increased eye movements during REM sleep was also characteristic of PTSD in this meta-analysis. Overall, these objective measures of sleep disruption corroborate subjective reports of sleep complaints.

Growing evidence points to a bidirectional relationship between daytime and nighttime symptoms of PTSD, suggesting that distinct sleep-related mechanisms may be involved in PTSD. Researchers have shown sleep disturbances occurring early after trauma exposure increase the risk of developing long lasting PTSD (van der Kolk et al., 1984; add: Harvey, 1999; Mellman, Pigeon, Nowell, & Nolan, 2007). Seventy-two percent of people in a study done by Harvey & Bryant (1998) who experienced sleep disturbances within the first month after trauma exposure developed PTSD at follow-up assessments. These findings have led some researchers to question whether sleep disturbances are simply symptoms of PTSD, or if they constitute a risk factor for the onset of the disorder (Lavie, 2001). In people with PTSD, sleep disturbances are associated with worsening of daytime PTSD symptoms, as well as with increased suicidality and increased severity of depressive symptoms, alcohol misuse, and poor perceived physical health (Krakow, et al., 2000; Clum, Nishith, & Resick, 2001; Saladin, Brady, Dansky, & Kilpatrick, 1995). In addition, we now know that PTSD sleep symptoms are highly resistant to first-line treatments of PTSD even if daytime symptoms are improved with selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), exposure therapy, or eye movement desensitization and reprocessing (Albucher & Liberzon, 2002).

We know sleep plays an important role in maintaining our physical and mental homeostasis (Borbely & Achermann, 1999; Van Cauter & Spiegel, 1999). Inadequate sleep

patterns can hinder mental health by decreasing cognitive performance, and increasing mood and emotional distress (Manocchia, Keller, & Ware, 2001). Poor sleep can also decrease the quality of physical health as well (Strine & Chapman, 2004). However, the role of sleep disturbances in the development and persistence of PTSD has not been closely and prospectively studied and probed.

### **1.1.2 Hypotheses**

Hypotheses attempting to understand the relationship between the sleep and daytime symptoms of PTSD are scarce. Some researchers have hypothesized that the underlying mechanism is biological in nature, and suggest that a dysfunction in the mechanisms of rapid-eye movement (REM) sleep following trauma exposure may contribute to PTSD (Jukie et al., 1999). For instance, Mellman and colleagues suggest that a disruption in REM sleep is caused specifically by insomnia and nightmares, and is believed to increase one's risk of developing PTSD after trauma exposure (Mellman, Bustamante, Fins, Pigeon & Nolan, 2002).

Others have hypothesized that the mechanism underlying the relationship between sleep and daytime symptoms of PTSD is more psychological and behavioral in nature. These researchers suggest that sleep-related anxiety most likely is the causal link between sleep and PTSD (Inman, Silver, & Doghramji, 1990). According to this theory, when sleep disturbances like nightmares and insomnia follow trauma, a conditioned anxious reaction towards sleep is created, leading to sleep avoidance, and to exacerbation of daytime symptoms.

In support of the latter, previous studies have shown that individuals with PTSD who also suffer from nightmares and insomnia are more likely to report sleep-related anxieties before bedtime, including fear of going or returning to sleep, and fear of being in the dark (Inman,

Silver, & Doghramji, 1990). It is important to note that while these hypotheses are both plausible and not mutually exclusive; little research has been done to directly test these hypotheses in human studies.

## **1.2 HUMAN LIMITATIONS IN PTSD RESEARCH**

Studying sleep may give researchers and clinicians further insight into the specific role sleep plays in the development and maintenance of this pervasive disorder. Specifically, several researchers have proposed that the role of sleep disturbances early following trauma exposure should be closely investigated, and may be a causal pathway for onset and persistence of the disorder, rather than just a diagnostic symptom as they are currently considered.

However, studying the temporal relationship between sleep disturbances and daytime symptoms of PTSD immediately after trauma exposure in humans is difficult. Obtaining data during the crucial time window between immediately after trauma exposure and when the patient seeks treatment. The observation that treatments of sleep disturbances improve daytime symptoms suggest that sleep-related mechanisms contribute to daytime PTSD symptoms and other poor psychological outcomes, but these findings are preliminary and must be researched further.

Currently there are limited means for observing this development in humans because observational studies are cross sectional and prevent researchers from tracking the development of PTSD symptoms across the sleep-wake cycle. The ideal experimental study would be a longitudinal design that allows researchers to study people before exposure to a trauma to carefully determine their baseline sleep characteristics, and study them at several different points



after some have been exposed to a traumatic event. This would give them the ability to simultaneously track changes in sleep disturbances pre- to post-trauma exposure, while observing whether the person begins to experience any additional daytime or nighttime symptoms of PTSD at any point after trauma. Most importantly, this kind of study allows researchers to record baseline measurements before trauma; knowledge current research does not have. With this kind of design, researchers could see if sleep disturbances occurring following trauma exposure cause the onset of daytime symptoms, or vice versa.

The limitations of human research that exist in PTSD and sleep research thus far have challenged researchers to find a treatment that will not just be a symptom reducer for PTSD symptoms across the sleep-wake cycle, but one that will emerge as a cure. PTSD so far has been resistant to treatments, but the available treatments do not target sleep disturbances as well as the disorder's daytime symptoms. In addition, studying the developmental course of PTSD across the sleep-wake cycle is vital for further research but difficult, at best, to do with human models due to methodological (selection bias may exist because less affected people may be more likely to participate in studies), logistical, and ethical considerations, as well as the lack of experimental control over the occurrence of trauma. Therefore, the combination animal models and longitudinal and clinical human studies is necessary to advance the current understanding of PTSD.

### **1.3 USING ANIMAL MODELS TO STUDY SLEEP IN PTSD**

Several animal models of PTSD have been established (Shin & Liberzon, 2010; Yehuda & Antelman, 1993), and some have been used to study sleep disturbances in PTSD. In recent novel

studies on the extinction of a conditioned fear in animals, rapid eye movement (REM) sleep disruption (more specifically, a decrease in REM) decreases the animal's extinction learning towards cue stimuli (Silvestri, 2005). An anxiety animal model using BALB/ cJ mice observed REM changes after only a single trial of fear conditioning and found almost immediate decreases in slow wave sleep (Sanford, Fang. & Tang, 2003). These results indicate that REM sleep is highly affected by responses of fear in animals. In terms of NREM sleep, an animal fear conditioning model using mice conducted by Hellman & Abel (2007) found that NREM sleep, rather than REM sleep, actually increased during the 24 hours after conditioning. They also found that mice spent more time awake after fear conditioning training.

Due to the high level of experimental control offered with animal models, researchers can use a classical fear conditioning model to elicit a conditioned fear response from a brief stressor to induce PTSD- like symptoms in rats and mice much similar to what we see in humans (Pawlyk, Jha, Brennan, Morrison & Ross, 2005; Hamner et al., 1999). By simulating PTSD-like symptoms in rats and mice through fear conditioning, we can observe the mechanisms underlying the relationship between sleep disturbances and PTSD-like symptoms, and ultimately use these findings and observations to inform human studies.

Animal models offer a unique level of experimental control that is not possible with humans. With mice specifically, there is the ability to control and manipulate the animal's genetic makeup, in addition to past experiences of stress exposure. In addition, animal models provide a unique platform to test novel treatments that cannot be tested in humans. If the nature and mechanisms of this link can be established in animals, this information can then be translated back to humans. By better understanding the mechanisms of the relationship between sleep and

PTSD, researchers can identify new treatments to alleviate both sleep and daytime symptoms of PTSD.

### **1.3.1 Fear conditioning animal model**

Although little PTSD research has been done on humans regarding fear conditioning and extinction, it has been proposed that the sleep disturbances associated with the disorder may disrupt the learning related to trauma exposure. A fear conditioning paradigm consists of an initially neutral stimulus [the conditioned stimulus (CS)] that is paired with an unlearned aversive stimulus [the unconditioned stimulus (UCS)] and repeatedly presented together until the pairing elicits an expected conditioned response (CR) (e.g. physiological responses in mice). This is repeated until the CS presented alone without the UCS will elicit the CR (which resembles PTSD- like symptoms). Extinction is defined as the learning of a new memory about the fearful CS/UCS association by presenting the CS repeatedly without the UCS after conditioning; therefore reducing the CR by weakening the learned stimulus association (Pace-Schott et al., 2009). It is possible that nightmares and insomnia facilitate recurrent reminders of trauma (UCS) in those with PTSD, and thus, people may be sensitized to trauma reminders and fear responses. This sensitization is believed to lead to avoidance behaviors towards sleep which causes an insomnia-like pattern to persist, ultimately maintaining the existing fear (CR). Extinguishing the association between the UCS and the CS does not erase the maladaptive memory associated with the trauma, but creates a new, more adaptive memory that can inhibit conditioned fear responses in the future (Pace- Schott et al., 2009; Quirk, Garcia, & Gonzalez-Lima, 2006).

Studies have shown that rats and humans share the same brain structures that are involved in fear conditioning, further supporting use of animal models as a viable alternative for experimentation (see MacLean, 2007 for review). More specifically, the ventromedial region of pre-frontal cortex (vmPFC) in both the human and rat brain are not completely homogeneous because the human brain is far more developed. However, there are several areas in the vmPFC involved in fear acquisition that both rats and primates (including humans) do share to perform the same functions (Milad, Rauch, Pitman, & Quirk, 2006). The amygdala is another area of the brain that is vital to study with fear conditioning because its primary function is processing memory and emotional reactions. Research has shown that damage to this area in the brain decreases the ability to produce an emotional response resulting from exposure to conditioned stimuli (Phillips & LeDoux, 1992).

This is all compelling evidence for the use of animal models to study fear conditioning, however problems do exist when using animal as subjects. To date, animal fear conditioning models have relied solely on behavioral measures of freezing recorded before, during and after conditioning (not physiological); a measure that cannot be easily translated back to human fear reactions. Other measures such as electromyography (EMG), heart rate, and blood pressure, however can be used in both animal and human subjects.

#### **1.4 TRANSLATIONAL RESEARCH: A NEW MURINE SLEEP-FOCUSED MODEL OF PTSD**

Translational research is a subset of research that is used in the biological, behavioral, and social sciences that not only helps science to understand relevant fear related processes in animals but

allows for the transference of knowledge acquired from basic sciences and methods to clinical and/or real-life situations in humans (Rosen, 2006). This unique kind of research is what has allowed researchers to study aspects of PTSD-like reactions in animals that is not possible to do with the human population. Carefully done research on mice, for example, can produce findings that are able to be translated back to humans and play a vital role in the understanding and treatment of some pervasive mental disorders, including PTSD. Our translational research is focused on developing and testing a novel fear conditioning animal paradigm that can ultimately provide insight into the link between sleep disturbances and daytime PTSD symptoms in humans.

Unlike previous research that has primarily used rat models, our model uses mice. The main advantage of mice is the possibility to use knock-out strains to probe the contribution of genetic factors in the relationship between sleep and PTSD. In mice researchers can also obtain objective measures of fear conditioning from heart rate, blood pressure and measures of adrenaline that are analog to measurable, objective indices of fear responses in humans.

My study takes place as the second part of a 2 part experimental protocol that serves to validate a novel experimental fear conditioning model focused on sleep disturbances relative to other established fear conditioning models of PTSD. This two part study also included physiological measurements of fear reactions such as heart rate and blood pressure. These physiological measurements were done to supplement traditional behavioral models freezing and have the advantage of being easily translated to humans for future studies.

In the first experiment, we tested a novel CS using mTH as the CS in mice. This experiment needed a conditioned stimulus that was detectable but not anxiogenic during wakefulness to use as the CS, so that it could be re-presented during sleep without awakening the

mice. For this purpose, a mild transient hypercapnia (mTH; 2.5% increase in CO<sub>2</sub> level) was chosen due to its effective use in previous animal research and was paired with a foot shock (Campen, et al., 2005) but has never been used for the purpose of fear conditioning.

Unique to this experiment was our ability to obtain continuous physiological measurements of heart rate, blood pressure, muscle tone (with electromyography), and brain activity (using electroencephalography) along with continuous monitoring of sleep-wake parameters over 24-hour periods before and after fear conditioning. As mentioned above, these physiological measures can be easily translated to indices of fear responses in humans (e.g., such as racing heart, and sweating palms) than the traditional behavioral measure of freezing.

Results from Experiment 1 showed no change in the blood pressure of the mice directly before shock, suggesting the possibility that this physiological measure may be highly resistant to mTH exposure. When compared to pre-conditioning measurements, change in EMG activity was not significant during the first and second stimulus pairings, but by the 3<sup>rd</sup> mTH/ shock pairing a near significant trend towards a decreased average EMG activity was observed. This may suggest that with the inclusion of additional stimulus pairings we would see a continued decrease in EMG activity maintained in the mice. Changes in heart rate were observed directly before shock in the form of a sudden decrease in heart rate from baseline measurements. This dramatic decrease in heart rate is known as a Bradycardic response.

In terms of changes to the sleep architecture of the mice, no changes were observed across all sleep parameters when comparing the 24 hours pre- conditioning to the 24 hours post-conditioning. However, when comparing 5 hour sleep pre- conditioning to the corresponding 5 hours post- conditioning a significant decrease in the percentage of time the mice spent in REM sleep was observed as well as a near significant trend towards an increase in NREM sleep.

Additional sleep architecture changes observed included a significant decrease in the number of times the mice went into REM sleep (REM sleep bout) and an increase in the average amount of time (in minutes) it took the mice to enter a REM sleep bout (REM latency). These results indicate that mice were experiencing decreases sleep time in the hours directly after shock exposure when compared to baseline sleep patterns, in a fear conditioning model using mTH as the CS.

#### **1.4.1 Experiment 2: Validating the new model of fear conditioning**

To validate our findings from Experiment 1 using mTH as the CS, a second experiment using a traditional tone as the CS was run. Tone was chosen because tones or lights are typically used as the CS in animal fear conditioning models. Comparing the effects of mTH/ shock and tone/ shock conditioning using the same behavioral and physiological measurements is an essential step in validating the new murine model of PTSD.

Determining if mTH can be an effective stimulus will be assessed by whether or not it can produce the same physiological and behavioral responses to fear when paired with a foot shock that the traditional tone does. If mTH proves to be effective we can determine that it is salient enough to be used in fear conditioning. The newly validated model would provide novel opportunities for studying and probing sleep in relation to PTSD, including studying the effects of CS presentation *during sleep*, to observe sleep disturbances caused by sleep induced CS presentations, and/or whether re-presentation during sleep facilitate or interferes with fear extinction. CO<sub>2</sub> has been used to study fear reactions in humans and it may be possible to develop a paradigm that uses CO<sub>2</sub> as a CS for human studies in the future (Babson, et al. 2009).

## **2.0 METHODS**

Our paradigm consisted of two identical experimental protocols. Aside for the difference in the CS, the experimental protocol used for Experiment 2 was identical to that used in Experiment 1. This paper will focus only on the methods from Experiment 2 using tone as the CS paired with a mild foot shock. The purpose of Experiment 2 was to confirm the use of mTH as an acceptable conditioned stimulus to use during fear conditioning (Experiment 1).

### **2.1 SUBJECTS/ ANIMALS**

FVB/nJ strain mice were used from the Jackson Laboratory in Bar Harbor, Maine. This particular strain was chosen due to its higher levels of activity and anxiety. Nine 10- week old male mice were used and weighed approximately 27 grams at the time of surgery. After surgery, all mice were housed in individual chambers and had continuous access to food and water during a 6 day recovery period. All mice were kept on a 12:12 hour light/ dark cycle with the light cycle beginning at 8 a.m. and experimental protocols beginning at 9am. .

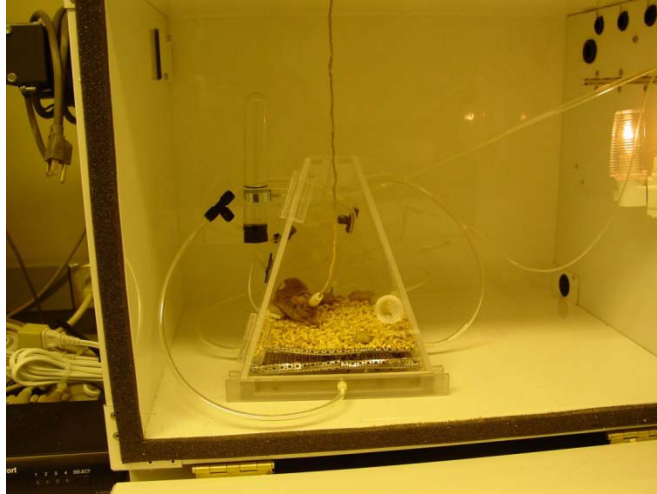


## **2.2 SURGICAL PREPARATION & CATHETERIZATION**

Surgery was done by a trained technician. During the surgical process, EEG and EMG electrodes were instrumented into the scalp and neck of each mouse to assist to determining the movement levels of the mice. Following instrumentation and a 6 day recovery period, a catheter was inserted into the femoral artery of each mouse for the purpose of taking blood samples and obtaining measures of heart rate. Catheterization was done 4 days prior to the experiment to allow for a sufficient amount of recovery time before training. This recovery period was important for the mice to adjust to moving around freely with the catheter attached to a swivel. The number of recovery days was determined to be sufficient based on previous animal research involving surgery. The recovery period was considered successful and the mouse was cleared to go through the experimental protocol if there were no observable health problems with the mouse as well as any discomfort or damage to the catheter.

## **2.3 APPARATUS**

All mice were housed and trained in a custom made pyramidal chamber placed in a light controlled and sound dampening box chamber (Figure 2.1).



**Figure 2.1: Housing and experimental chamber for mice for experimental protocols**

For tone production, a Tone/ Noise Generator (model A69-20) from Coulbourn Instruments was used to deliver a 2400Hz, 80dB tone. A literature review on tone as a CS for fear conditioning indicates this specific tone has been commonly used. A Radio Shack Digital Sound Level Meter was also purchased to regulate the decibel reading from the tone generator. The shock producing system used was the Precision Regulated Animal Shocker with an electric floor shock grid (model H13-15) from Coulbourn Instruments in Whitehall Pennsylvania. All blood samples taken were spun down using a Forma Refrigerated MicroCentrifuge (model 5522) from Thermo Electron Corporation and were stored and frozen in a Thermo Electron Corporation Forma- 86C ULT Freezer (model 900 series) for later catecholamine analysis.

## **2.4 EXPERIMENTAL PROTOCOL**

To be consistent with Experiment 1, Experiment 2 followed a protocol identical to the one used in Experiment 1. In Experiment 1 the decision to develop the study protocol was made to be

consistent with previously published studies on fear conditioning animal models (Jha et al., 2005). This novel fear conditioning paradigm using mice focused on measuring the effect shock exposure has on the stress level of mice. This effect was measured in terms of nuchal EMG freezing as a result of shock exposure (complete immobility aside from regular breathing), and was estimated by nuchal (neck) EMG activity. Changes in heart rate and blood pressure, as well as alterations to sleep- wake patterns (specifically in NREM and REM sleep) were also monitored and assessed. We anticipated the physiological measurements from the second experiment using tone would be comparable to measurements from Experiment 1 using mTH. Evidence of this pattern will serve as validation for future use of mTH as an effective CS for fear conditioning.

We specifically chose to measure the blood pressure and heart rate of the mice because we felt these physiological measures can be easily translated to humans and have been shown to predict chronic stress in human responses (Pitman, et al. 2002).

#### **2.4.1 Experiment 2**

Day 1 served as a pre- conditioning 24 hour sleep study beginning at 8 a.m. the day before fear conditioning training. This light dark cycle was used for convenience of doing the experiment during the day. Day 1 served to obtain baseline measurements of sleep patterns and the mouse was left undisturbed for this period. On Day 2, the experimental procedures were conducted. Shortly after 8:00 a.m. a blood sample of forty microliters was drawn from the mouse and then the housing/ training chamber was cleaned. The blood sample was then placed into the centrifuge and spun down at a rate of 8000 rpms for 4 minutes at 4 degrees Celsius. Once the serum in the sample had separated, it was extracted into a separate tube with a pipette and placed

into a freezer for later analysis. The spun down sample was then mixed until homogenous with 20 microliters of 100U heparin solution and resuspended back into the mouse. Blood samples were taken to assess the plasma levels of the catecholamines, adrenaline and noradrenalin. Although taken during the protocol, analysis of blood samples is not included in this paper.

The protocol began at 9:00 a.m. when the first CS alone test was administered. Each mouse was presented a 2400Hz, 80 dB tone alone with no presentation of shock for thirty seconds 5 times in succession with a 2 minute recovery period in between each trial.

A second blood sample was then withdrawn immediately following the CS alone test and followed the same method used for the first blood sample. At 10:00 a.m. the mouse received its first presentation of the tone/ foot shock stimulus pairing. Tone was presented for 30 seconds and immediately after its termination five consecutive 0.5 mA foot shocks for 0.5 seconds each followed (Table 2.1).

**Table 2.1: Tone/ shock delivery schedule for conditioning used during Experiment 2. This schedule occurs at 10:00am, 12:00pm and 2:00pm on Day 2. Each stimulus pairing is followed by a 2 minute recovery period.**

| <b>Tone/ Shock Delivery Schedule (10:00, 12:00, and 2:00 on Day 2)</b> |                    |   |
|--|--------------------|---|
| <b>Tone (30 seconds)</b>   | <b>→</b>           | <b>5 Foot Shocks (0.5mA) 0.5 seconds each</b> |
|  | <i>(2 minutes)</i> |   |
| <b>Tone (30 seconds)</b>   | <b>→</b>           | <b>5 Foot Shocks (0.5mA) 0.5 seconds each</b> |
|  | <i>(2 minutes)</i> |   |
| <b>Tone (30 seconds)</b>   | <b>→</b>           | <b>5 Foot Shocks (0.5mA) 0.5 seconds each</b> |
|  | <i>(2 minutes)</i> |   |
| <b>Tone (30 seconds)</b>   | <b>→</b>           | <b>5 Foot Shocks (0.5mA) 0.5 seconds each</b> |
|  | <i>(2 minutes)</i> |   |
| <b>Tone (30 seconds)</b>   | <b>→</b>           | <b>5 Foot Shocks (0.5mA) 0.5 seconds each</b> |

After the stimulus exposure a third blood sample was drawn. At 12:00 p.m. the mouse once again received tone for 30 seconds followed by the 5 foot shocks followed by another blood draw. This procedure was then repeated identically a final time at 2:00 p.m. At 3:00 p.m. the mouse again received 5 presentations of tone alone without foot shock for 30 seconds with a 2

minute recovery period in between each presentation. This final tone alone was crucial to make sure if we did begin to see a fear response that it was caused by the tone/ shock pairing and nothing else. Although no shock followed the tone increase at this point, if the mouse was successfully fear conditioned it will still prepare for shock in the presence of tone as a result of the mouse learning the association between the two stimuli. The last blood sample was taken after the post- conditioning tone alone test, making a total of 6 samples taken throughout the day. Data analysis from blood samples are currently in progress and currently not available. This concluded the experimental protocol of Day 2 (Table 2.2).

**Table 2.2: Three day experimental protocol using tone/shock**

|              |                       |   |                |
|--------------|-----------------------|---|----------------|
| <b>Day 1</b> | 8:00 a.m. – 8:00 a.m. | <b>24 hour Pre- Conditioning<br/>Sleep Study</b>      |                |
|              |                       |   | ← Blood Sample |
|              | 9:00 a.m.             | <b>Tone (30 seconds)</b>                              | x 5            |
|              |                       |   | ← Blood Sample |
|              | 10:00 a.m.            | <b>30 second tone<br/>0.5 mA Foot Shock (0.5 sec)</b> | x 5            |
|              |                       |   | ← Blood Sample |
| <b>Day 2</b> | 12:00 p.m.            | <b>30 second tone<br/>0.5 mA Foot Shock (0.5 sec)</b> | x 5            |
|              |                       |   | ← Blood Sample |
|              | 2:00 p.m.             | <b>30 second tone<br/>0.5 mA Foot Shock (0.5 sec)</b> | x 5            |
|              |                       |   | ← Blood Sample |
|              | 3:00 p.m.             | <b>Tone (30 seconds)</b>                              | x 5            |
|              |                       |   | ← Blood Sample |
| <b>Day 3</b> | 3:00 p.m. – 3:00 p.m. | <b>24 hour Post-Conditioning<br/>Sleep Study</b>      |                |

After conditioning, all mice were housed in the same chamber where the shock training occurred for the next 24 hours to avoid any influence of environment on FC. This final 24 hours (Day 3) served as a post conditioning sleep study that allowed us to observe any measurements

of the sleep- wake patterns of the mice that differed from the baseline measurements obtained on Day 1.

## **2.5     PHYSIOLOGICAL & SLEEP RECORDINGS**

Experiment 2 measured the same recordings that were measured in Experiment 1. Both electroencephalogram (EEG) and electromyography (EMG) electrodes were chronically implanted into the scalp and the back of the neck of each mouse. Electrical brain activity was measured using electroencephalogram recordings. Electrical muscle activity was measured from electromyography recordings from the nuchal (neck) muscle to assist with determining freezing time. Measuring the EMG of the mice using neck muscle has been shown in previous research to be an effective method of scoring fear behavior and just as effective as traditional visual based (behavioral) scoring methods (Steenland & Zhou, 2009). Both the EEG and EMG recordings using an EEG and Polygraph data recording system provided the ability to determine the sleep state of the mice at any given point in time.

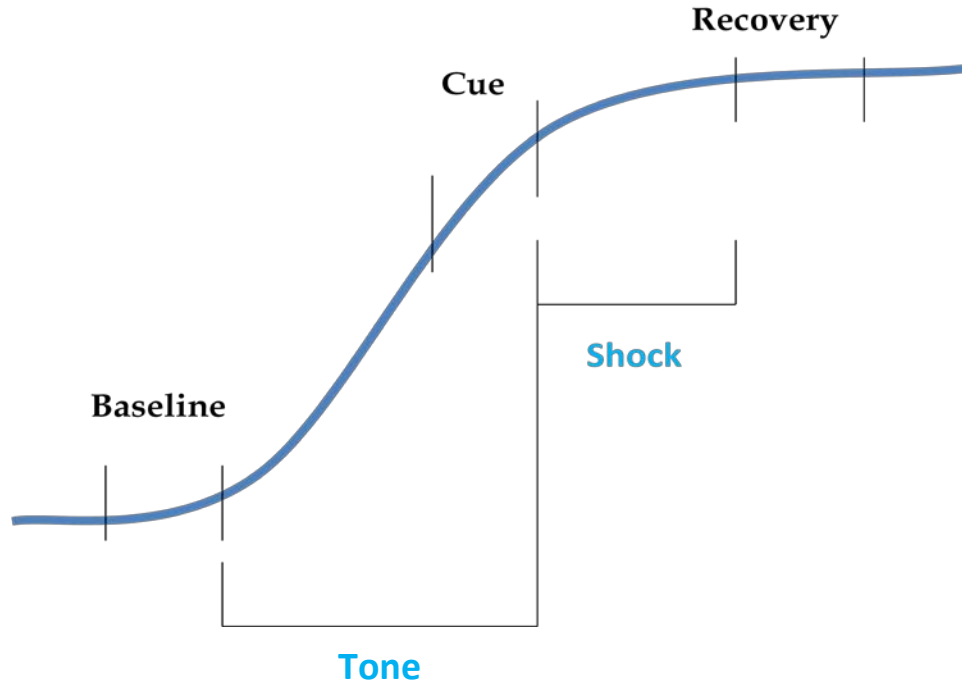
Absolute heart rate and blood pressure were also measures of interest in our protocol and were recorded using the WinDaq software program before, during and after fear conditioning. All measures were continuously recorded from the catheters in the mice from the beginning of the 24 hour pre- conditioning sleep study on day 1 to the end of the 24 post- conditioning sleep study on Day 3.

## **2.6 DATA ANALYSIS**

Descriptive statistics of mean and standard error were calculated for all measures of interest. To determine significance, two- tailed paired t-tests were conducted to be conservative and to assess differences across pre and post experimental conditions within each experiment on physiological measurements and sleep recordings.

### **2.6.1 Heart Rate & Blood Pressure**

Absolute heart rate (HR) and mean arterial blood pressure (BP) were measured during three time intervals (baseline, cue, and recovery) that were thought to be critical in determining if the mice were in fact showing the expected conditioned fear response. First, mean HR and BP measurements were recorded from the 10 seconds before the presentation of the CS (either mTH or tone), this was called the baseline period. Secondly, mean HR and BP were analyzed during the last 10 seconds of CS exposure directly before shock onset. This was referred to as the cue period. Finally, the same averages were recorded from the 10 seconds directly after the termination of shock, called the recovery period. These 3 periods were measured for both CS alone presentations and all 3 stimulus pairings on Day 2. All together there were 5 different stimulus presentations during the protocol with 3 measures of interest (1 for each time interval), making for a total of 15 measurements (Figure 2.2).



**Figure 2.2: Three time periods of interest throughout experimental protocol. Baseline refers to the 10 seconds directly before tone onset. Cue period refers to the final 10 seconds of tone presentation, directly before shock onset. Recovery period refers to the 10 seconds directly after shock termination.**

## 2.6.2 EMG Activity

Nuchal EMG activity was measured during the entire 30 second duration of the tone before the onset of shock. Mean EMG activity was of interest over 7 different time periods; during quiet wakefulness and NREM sleep, in addition to all 5 stimulus presentations. These measurements would help to determine if the expected behavioral fear response from the mice (freezing) was occurring. Although it is important to note this study does not include behavioral measurements of freezing due to physiological measurements as the focal point.



### **2.6.3 Sleep- Wake Analysis**

Arguably, the most important measurements analyzed were the sleep- wake states recorded before and after conditioning. Sleep recording began 24 hours before the FC protocol and continued until 24 hours after conditioning. The 24 hours of sleep pre- conditioning served as baseline measurements of sleep patterns and were compared to sleep patterns observed during the 24 hours post- conditioning. A sleep state software program measured sleep parameters in 10 second epochs for several different sleep parameters of interest including, total percentage of time spent awake, and in NREM and REM sleep, number of times the animal goes into NREM and REM sleep (sleep bouts), average length of each sleep bout, REM latency, and propensity to fall back to sleep after an arousal.

After comparing parameters recorded during the 24 hours of sleep pre and post conditioning we then compared sleep during the 5 hours immediately after fear conditioning (4pm- 9pm) to the corresponding hours during the pre- conditioning sleep recordings (4pm- 9pm the day before conditioning). The 5 hour period was selected arbitrarily and was done to observe if sleep differed shortly after conditioning (when trauma may be more remembered) compared to when the animal has had more time to recover (the entire 24 hours after).

## **3.0 RESULTS**

### **3.1.1 Experiment 1**

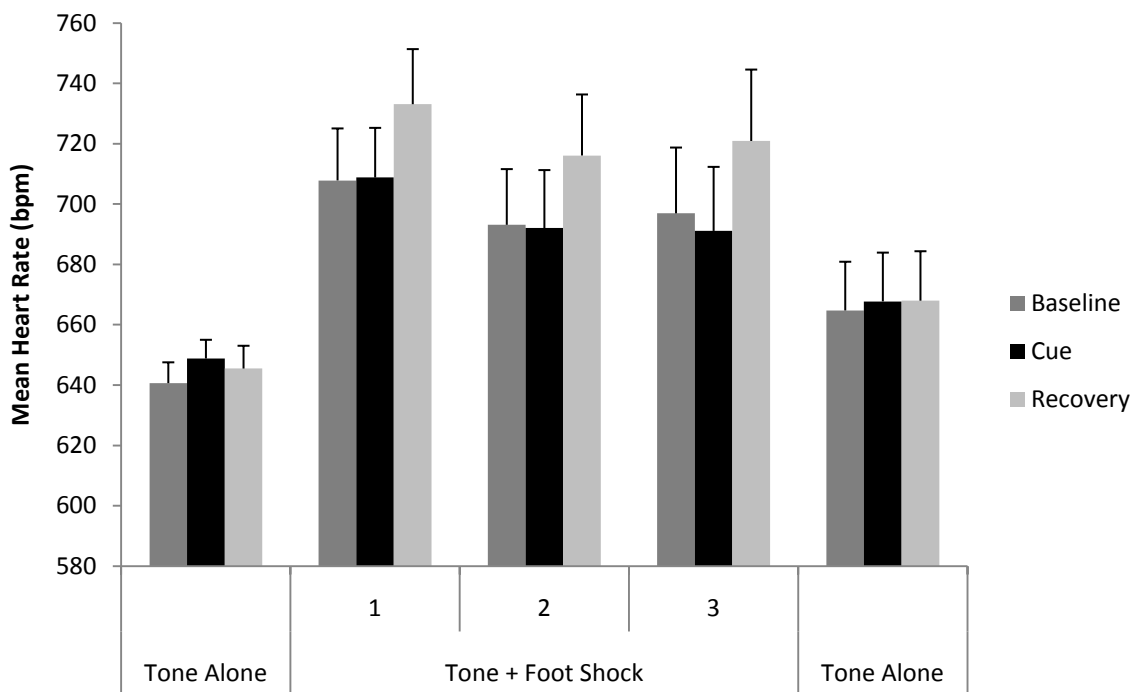
For completeness and comparison, results from Experiment 1 can be found in Appendix A.

### **3.1.2 Experiment 2**

Based on results obtained in Experiment 1, we made several hypotheses for Experiment 2. We hypothesized we would see a Bradycardic response (sudden slowing of the heart) in the mice during the 10 seconds directly before shock (the cue period). We also expected to see no significant changes to the blood pressure of the mice as well as minimal changes to EMG activity. In terms of sleep- wake patterns we hypothesized there would be no changes to sleep architecture in the 24 hours of sleep post- conditioning when compared to baseline measurements. We did however expect to see significant changes to the sleep patterns of the mice (specifically a decrease in REM sleep and increase in NREM sleep) when comparing the 5 hours pre- conditioning to 5 hours immediately following conditioning. We hypothesized this because we felt the sleep patterns of the mice were more affected immediately following shock exposure.

When compared to tone alone, mean heart rate was measured from the baseline to the cue interval and no evidence of bradycardia was found (Figure 3.6). These results were surprising

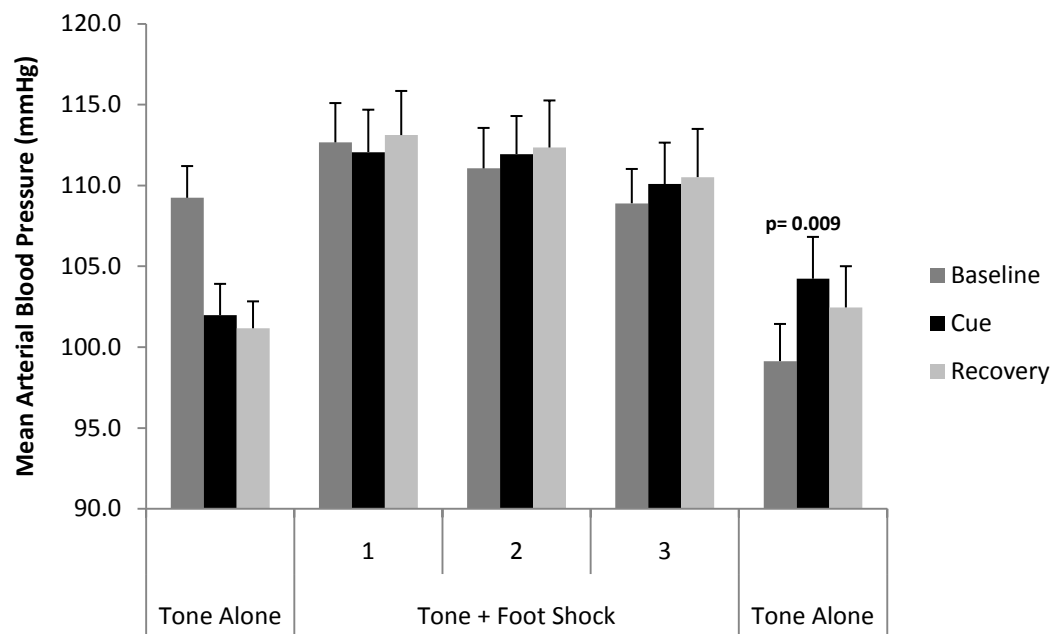
considering such a strong bradycardic response developed and maintained itself after conditioning in the majority of the mice in Experiment 1. The lack of evidence for a decrease in heart rate in preparation of shock may indicate that mTH affects heart rate measures in a way that tone does not.



**Figure 3.1: Mean heart rate response (bpm) over type of stimulus presentation for 3 critical time periods during stimulus conditioning. Error bars refer to the positive standard errors of the mean. P- values were calculated using paired t-tests and represent comparisons between baseline, cue and recovery periods.**

In terms of absolute blood pressure it was hypothesized that similar to Experiment 1, there would be no significant changes in post conditioning. Figure 3.7 shows the mean blood pressure response during stimulus exposure. Our hypothesis was proven correct; there were no significant changes in blood pressure before or during conditioning. However, unique to Experiment 2, post conditioning measurements of increased BP were found to be significant from the baseline to the cue period,  $p = 0.009$  (Figure 3.7). It is difficult to make any conclusions

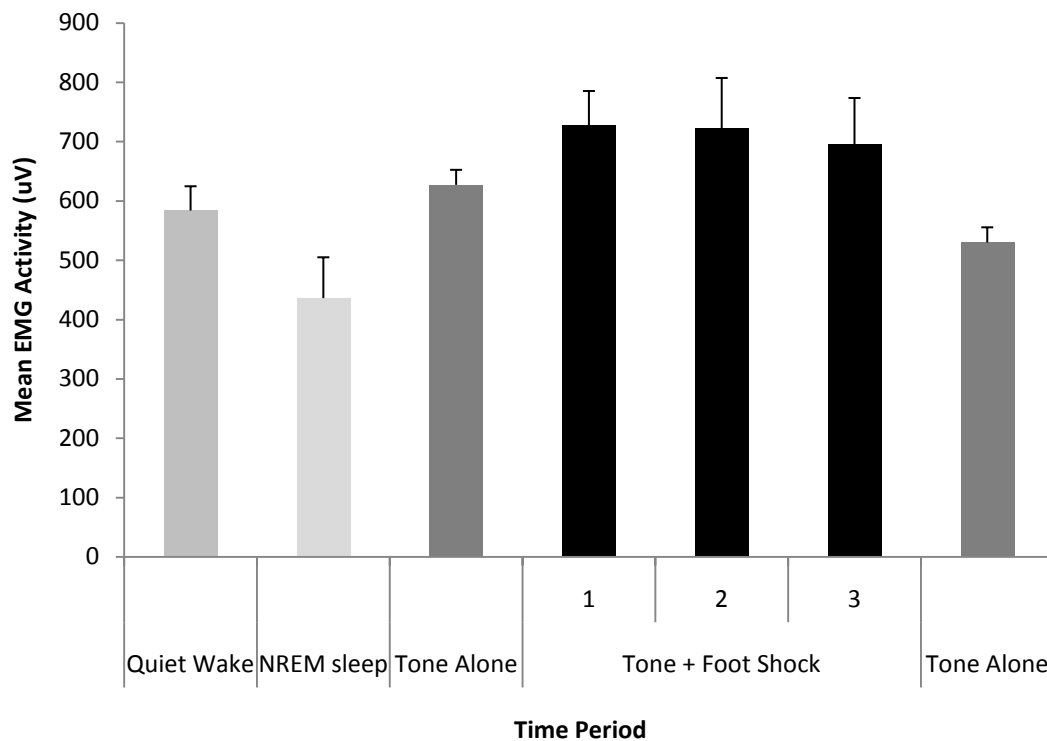
about this isolated post conditioning surge in blood pressure especially due to the small sample size. We cannot be certain if we were to continue presenting the tone/ shock pairing to the mice that this response would be maintained further. If this pattern would maintain itself with a larger sample of mice this will validate our original hypothesis that shock exposure will cause an increase in BP. As mentioned with blood pressure results from Experiment 1, it is possible blood pressure is highly resistant to responses of fear and that more trials of tone/ shock are needed to see changes in blood pressure.



**Figure 3.2: Mean arterial blood pressure (mmHg) over type of stimulus presentation. Blood pressure was measured during both tone alone and all tone plus foot shock pairings for 3 different time intervals during conditioning (baseline, cue and recovery). Error bars refer to the standard errors of the mean. P-values were calculated using a paired t-test and represent comparisons between the baseline, cue and recovery periods.**

In addition to absolute blood pressure and mean heart rate, nuchal EMG activity was a physiological measure of importance to assist in determining freezing. The mean EMG activity

was measured during the 30 second tone exposure for 7 different time periods. When compared to mean EMG activity to pre conditioning tone exposure, it was predicted there would be no significant difference in muscle activity level during the tone/shock pairings; therefore an absence of freezing.



**Figure 3.3: Mean EMG activity (uV) over 7 different time periods of interest before, during, and after conditioning. Measurements were taken during the presentation of a 30 second tone. Error bars refer to the standard errors of the mean. P- values were calculated using paired t-tests and represent comparisons between (1) pre- conditioning tone alone and tone + foot shock pairing, and (2) between tone + foot shock pairings and post- conditioning tone alone.**

Although mean EMG activity in Figure 3.8 shows a slight decrease in activity from the second to third tone/ shock pairing and was maintained during the post conditioning tone exposure, paired t- tests showed no significant trend in the data. We can conclude that measurements of EMG activity remained unaffected in both experiments 1 and 2 and could

possibly not be the best way to assess freezing due to lack of the more commonly used behavioral measurements of freezing in the study.

Based on observations of EMG activity during the post conditioning tone alone, adding additional tone/ shock pairings to the protocol may yield significant differences in muscle activity. Further subjects must be run with more exposures to assess this.

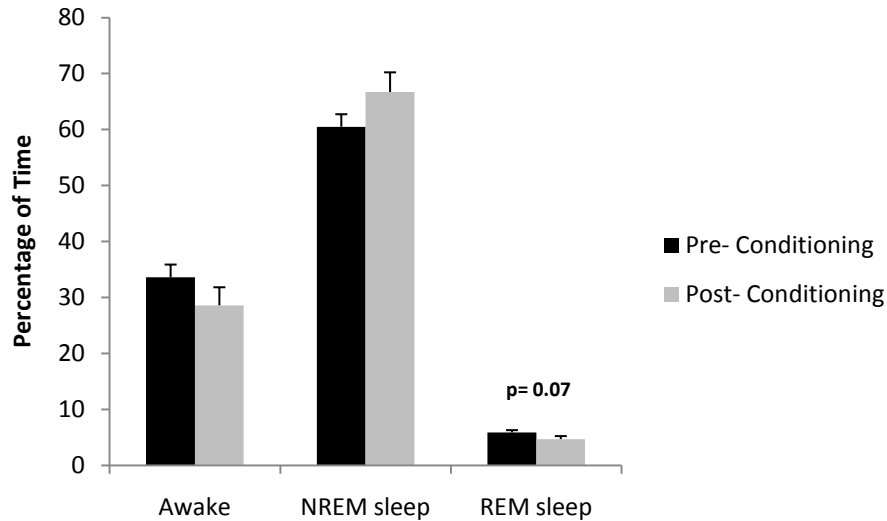
Finally, sleep states were continuously recorded beginning from the 24 hours directly before conditioning began and ending 24 hours after conditioning finished. It was hypothesized as in Experiment 1, that there would be no significant changes in sleep architecture when comparing sleep patterns from the 24 hours pre- conditioning to the 24 hours post conditioning. Figure 3.1 shows that our hypothesis was partially correct. The majority of sleep parameters measured during the 24 hours post- conditioning remained unchanged as seen in Experiment 1.

**Table 3.1: Comparison of sleep- wake parameters between 24 hours selected before conditioning and the 24 hours directly after conditioning. P- values were calculated using paired t- tests and represent comparisons between pre and post conditioning 24 hour sleep.**

|   | Pre- Conditioning | Post- Conditioning |                |
|---|-------------------|--------------------|----------------|
| <b>Time Awake:</b>  | 28.9 %            | 27.7 %             |                |
| <b>Time NREM Sleep:</b>                                   | 65.8 %            | 66.2 %             |                |
| <b>Time REM Sleep:</b>                                    | 5.4 %             | 6.2 %              | <b>p= 0.03</b> |
| <b>Total Sleep Bouts:</b>                                 | 89.9              | 87.8               |                |
| <b>Average Length of Sleep Bouts:</b>                     | 11.5 minutes      | 11.8 minutes       |                |
| <b>NREM Sleep Bouts:</b>                                  | 135.0             | 142.8              | <b>p= 0.05</b> |
| <b>Average Length of NREM Sleep Bouts:</b>                | 6.6 minutes       | 6.7 minutes        |                |
| <b>REM Sleep Bouts:</b>                                   | 62.8              | 73.4               |                |
| <b>Average Length of REM Sleep Bouts:</b>                 | 1.1 minutes       | 1.1 minutes        |                |
| <b>Average REM Latency:</b>                               | 8.5 minutes       | 9.0 minutes        |                |
| <b>Propensity to fall asleep after arousal (average):</b> | 1.7 minutes       | 1.9 minutes        |                |

However, two significant differences were observed unique to Experiment 2. Total percentage of time the animals spent in REM sleep did in fact increase from 5.4% to 6.2%, a difference found to be significant through paired t- tests,  $p= 0.03$ . Also, in terms of NREM sleep, the number of NREM sleep bouts increased significantly,  $p= 0.05$ .

Due to significant changes in sleep patterns (specifically in NREM and REM) observed in Experiment 1 during the 5 hours directly after fear conditioning, it was predicted similar trends would occur after conditioning in Experiment 2. More specifically, it was predicted during the first 5 hours after conditioning, there would be a decrease in the percentage of time spent in REM sleep and the number of REM sleep bouts along with an increase in the percentage of time spent in NREM sleep and shorter duration of each NREM sleep bout. This study arbitrarily chose to look at 5 hour sleep in addition to the full 24 hours recorded. This was hypothesized because soon after exposure to trauma (such as shock), sleep patterns differ; possibly preventing the animal from staying asleep longer and entering restorative REM sleep. Figure 3.9 compares sleep- wake states observed during pre and post conditioning 5 hour intervals.

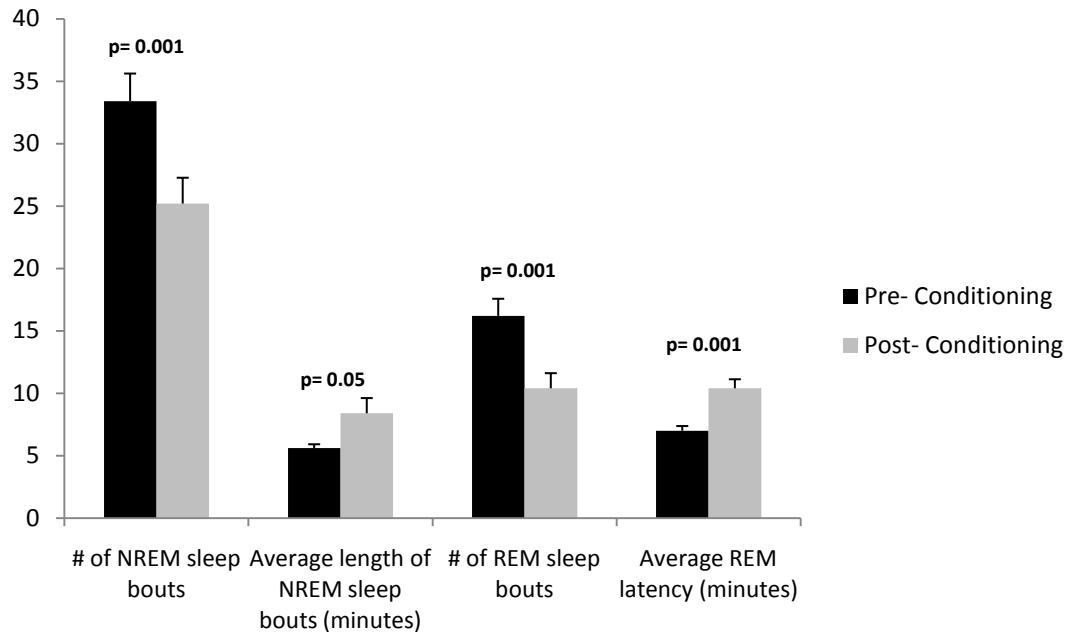


**Figure 3.4: Comparison of percentage of time spent in different sleep- wake states between 5 hours selected before conditioning and the 5 hours directly after the end of conditioning. The five hour ranges analyzed during both pre and post conditioning ran from 4pm to 9pm. Error bars refer to the standard error of all sleep measurements from the mean. P- values were calculated using paired t-tests and represent a comparison between pre and post conditioning 5 hour sleep.**

In terms of total percentage of time spent in NREM and REM sleep our hypotheses were partially correct. As expected, there was a near significant decrease in REM sleep observed,  $p=0.07$  showing a strong trend in the data. It is important to note that the sample used in Experiment 2 was much smaller than Experiment 1. It is highly likely that with the addition of more mice to the sample the difference in REM would become significant. Surprisingly, although an increase in NREM sleep was observed, it proved not to be significant, contradictory to our predictions. It is likely that with a larger sample size that this measure could become significant.

Unlike results found in Experiment 1, additional changes in sleep parameters were observed in the 5 hours post- conditioning of Experiment 2. Figure 3.10 compares the pre and post conditioning measurements of all other sleep parameter found to be significant.





**Figure 3.5: Comparison of several NREM and REM sleep parameters between 5 hours selected before conditioning and the 5 hours directly after the end of conditioning. The five hour ranges analyzed during both pre and post conditioning ran from 4pm to 9pm. Error bars represent the standard error of NREM and REM measurements from the mean. P- values were calculated using paired t-tests and represent a comparison between pre and post conditioning.**

Both the number of NREM and REM sleep bouts significantly decrease after conditioning,  $p = 0.001$ . This shows that overall the mice entered a sleep state less often. We also observed an increase in the average length of each NREM sleep bout that was found to be significant,  $p = 0.05$ . This indicates that although the mouse was going into NREM sleep less often, when it did the sleep bout was longer in duration. Spending a longer time in NREM sleep explains also why a significant increase in average REM latency was observed,  $p = 0.001$ . As a result of increase time spent in REM, it takes the mouse longer to enter REM that is if the mouse remains asleep during NREM without an arousal.

The effects tone/ shock exposure had after conditioning during Experiment 2 were much greater in terms of sleep than observed in Experiment 1 using mTH and shock. It is possible that tone is a stimulus that has the ability to alter sleep patterns post trauma in a way mTH cannot. Sleep changes in Experiment 2 support theories that trauma has negative effects on quality of sleep in mice. However it is important to note that while Experiment 2 saw significant changes in sleep, significant changes in physiological measurements were not observed. Experiment 1 showed mTH/ shock produced some physiological changes and some differences in sleep but not to the extent we saw in Experiment 2. It could be that these 2 stimuli can both be used effectively for fear conditioning but the measurements that they have effects on are different.

## **4.0 DISCUSSION**

The role of sleep disturbances in PTSD remain poorly understood, and prospective investigations in humans prove several methodological and ethical challenges. Animal models give researchers the opportunity to observe physiological and sleep changes that occur immediately post-trauma exposure that are not possible to induce and monitor in humans. Our study of a murine animal model for PTSD that consisted of two experiments, with Experiment 1 using a mTH, as the CS and Experiment 2 using a traditional tone. Both experiments followed identical protocols with the same stimulus delivery schedules, and a unique set of physiological and sleep measurements were conducted in both experiments.

The two aims in this study were to (1) obtain physiological measurements rarely studied in animal fear conditioning models that are commonly linked to fear responses before, during and after shock exposure while also monitoring behavioral responses like freezing and sleep states; (2) validate results obtained in Experiment 1 and the use of a mild mTH as a viable conditioned stimulus for fear conditioning by replacing the mTH with the traditionally used tone as the CS. By validating the use of mTH as a potential CS, we anticipate the use of a new paradigm to probe sleep in subsequent studies. This will allow us to study how the presentation of a fearful stimulus during sleep affects fear responses, including fear extinction. This may then serve as a paradigm to study the effects of nightmares and insomnia in PTSD patients.

In the present study, and by using an animal model with tone as the CS that has been commonly used to study fear conditioning, it was possible to record reliable and valid baseline physiological measurements to analyze and compare to identical measurements recorded using mTH and foot shock.

In Experiment 1, we found evidence of a strong bradycardic response to fear exposure in the mice that occurred directly before shock during CS exposure; indicating the mice were preparing for shock. Other physiological measurements of blood pressure and EMG activity remained unchanged post conditioning when compared to baseline measurements. Post-conditioning sleep measurements found an increase in NREM sleep and a decrease in REM sleep as expected. Decreases in REM sleep bouts and an increased time to enter REM sleep were also observed. These results are strong evidence for fear conditioning having an impact on the sleep patterns of mice during the first 5 hours after conditioning.

Experiment 2, surprisingly showed no evidence of a decreased heart rate directly before shock exposure, nor were any significant changes in blood pressure and EMG activity observed. However, similar to results in Experiment 1, tone/ shock conditioning also resulting in a large impact on sleep parameters during the 5 hours directly following conditioning. A significant decrease in the total percentage of time spent in REM sleep was observed. Also similar to Experiment 1, there were decreases in both NREM and REM sleep bouts, and increase in the amount of time spent in NREM sleep as well as an increased REM latency. Hour by hour sleep analysis will be done in the near future on Experiment 1 & 2 sleep data to determine any differences in sleep patterns.

These results indicate that exposure to CS, which are used as proxy for traumatic stimuli, can result in sleep disruption; findings are promising for future research for PTSD using animal

models that can be translated back to humans. It is also possible that the physiological measurements of EMG activity and blood pressure in mice are more resistant to aversive stimuli, supporting the lack of changes in these measurements during both experiments 1 and 2. However, the bradycardic response present in Experiment 1 only is intriguing, and should be further investigated as a potential sensitive marker of fear response in the future.

Overall, the patterns observed in both Experiments 1 & 2 before, during and after conditioning across behavioral, physiological and sleep measurements partially confirm the major hypotheses of the study about post- conditioning changes in sleep patterns.

This promising novel murine model using mTH as a CS to study the role of sleep in fear responses and PTSD has many advantages. The high level of experimental control allows for manipulating the genetic makeup and past experiences; reducing the risk of any confounding variables that are often inherent to human studies. Using our novel animal model also allows for the collection of objective, physiological data during the critical period between trauma exposure and the onset of symptoms. These same physiological measurements can be conducted during wakefulness and sleep, so that our model will allow us to probe REM and NREM sleep in future studies and quantify and compare objective indices of fear response across the sleep-wake cycle. One limitation of animal models is the inability to gather subjective measures.

These results though promising come with some limitations. While preliminary, the findings from these experiments should be replicated with a larger sample of animals, and in mouse strains that are more commonly used for fear conditioning (such as the BALB/cJ and C57BL strains). Additional subjects are currently being added to Experiment 2 to ensure results are consistent and the unequal subject pool is not a confounding factor. Our study's use of a novel way to assess freezing through nuchal EMG activity can be useful our protocol lacks a

method to measure freezing behaviorally. Behaviorally measuring freezing is common in animal fear conditioning and the addition of it to our protocol may be necessary in the near future. Also, current this study contains no control group for comparison and will be added in the near future to the protocol. Another limitation wrote attention is the stable, unchanging environment the mice are housed and trained in throughout the protocol. This was done to avoid any confounding factors of context in our results but may not be representative for human conditions regarding trauma because humans rarely experience the same environment when exposed to what they are fearful of

If results remain consistent after making alterations to our protocol, we can accept mTH as an acceptable CS for fear conditioning, allowing for the presentation of a stimulus during sleep without awakening the animals; a significant step forward for PTSD research on sleep disturbances.

#### **4.1 IMPLICATIONS FOR FUTURE TREATMENT RESEARCH**

Understanding the etiology and patterns of the behavioral, physiological effects and sleep changes resulting from exposure to a traumatic stimulus is a necessary component for future animal PTSD models where the ultimate goal is to translate results to the human population with PTSD.

The importance of adequate sleep is becoming a larger focal point in research and the sleep disturbances associated with PTSD are gaining more support for being much more than a just a nighttime symptom of the disorder. Current, widely used first-line treatments for PTSD include pharmacological medications, exposure therapy, trauma focused cognitive behavioral

therapy (TFCBT), eye movement desensitization and reprocessing (EMDR) and group cognitive behavioral therapy, all of which were proven to be equally effective through a meta-analysis done by Benish, Imel, and Wampold (2010). These same treatments are all considered “trauma-focused” and have been ineffective in treating nighttime symptoms of PTSD due to lack of a “sleep-focused” dimension.

The high treatment resistance of nightmares and insomnia are evidence that a sleep disturbances after trauma may be facilitating the onset of PTSD daytime symptoms. Acknowledging sleep disruptions after trauma exposure as a factor for the disorder onset means the development of new, “sleep focused” prevention and treatments that are vital to make sleep disturbances less resistant to intervention and ultimately restore healthy sleep patterns before the onset of any daytime symptoms in the near future.

Right now, the most popular form of psychotherapy used to treat sleep disturbances in PTSD is cognitive-behavioral therapy for insomnia (CBT-Insomnia) and is considered to be the “gold standard for treating insomnia”. However, the effectiveness of CBT-Insomnia for the treatment of nightmares is not certain (Swanson, Favorite, Horin & Arnedt, 2009). The inability for this treatment to reduce both of the most commonly reported sleep disturbances linked to PTSD poses a significant problem that may make it an ineffective choice for those with PTSD. Image rehearsal therapy (IRT) and relaxation therapy are both psychotherapies that have been effective for the treatment of PTSD related nightmares. Prazosin, an  $\alpha$ -1 antagonist drug has shown in several replicated trials to be effective in reducing both sleep and daytime symptoms but the drug must be used continuously to maintain its benefits because symptoms return when dosage stops (Germain, 2009).

It is clear that (1) treatments commonly used for PTSD's daytime symptoms are "trauma focused" and do not alleviate pervasive nighttime symptoms and (2) among the current treatments shown to be effective in the treatment of insomnia and nightmares, each has been proven to be effective in the treatment of only 1 of these 2 sleep disturbances. A treatment has yet to be found that is "sleep focused" and is consistently effective for treating all sleep symptoms experienced by PTSD patients. The development of a sleep focused treatment made up of components of existing therapies that have been effective in reducing either nightmares or insomnia may be the answer. More specifically, preliminary results from recent research combining IRT with components of CBT- Insomnia have shown improvements in dysfunctional sleep and PTSD post treatment (Germain, Shear, Hall & Buysee, 2007). Swanson, Favorite, Horin, & Arnedt (2009) combined elements of the exposure, relaxation and rescripting therapies (proven to be effective for nightmare reduction) with components of CBT- Insomnia (including sleep restriction and stimulus control) into a treatment that was delivered to combat veterans in the form of group therapy sessions. Preliminary results showed improvements in both insomnia and nightmares, suggesting a combination treatment may be the answer for the reduction of sleep disturbances. It was also reported the treatment was not effective in alleviating PTSD's daytime symptoms.

Although research focused on treating post- trauma sleep disruptions in those with PTSD as a cause for the onset of the disorder's daytime symptoms is novel and scarce, more is known about the relationship between the 2 distinct symptom categories than before. Defining the exact relationship nightmares and insomnia share with PTSD's daytime symptoms will be a difficult task due to lack of ability to observe the development course of the disorder's symptoms and PTSD's high comorbidity with other psychological disorders and medical issues. However, animal

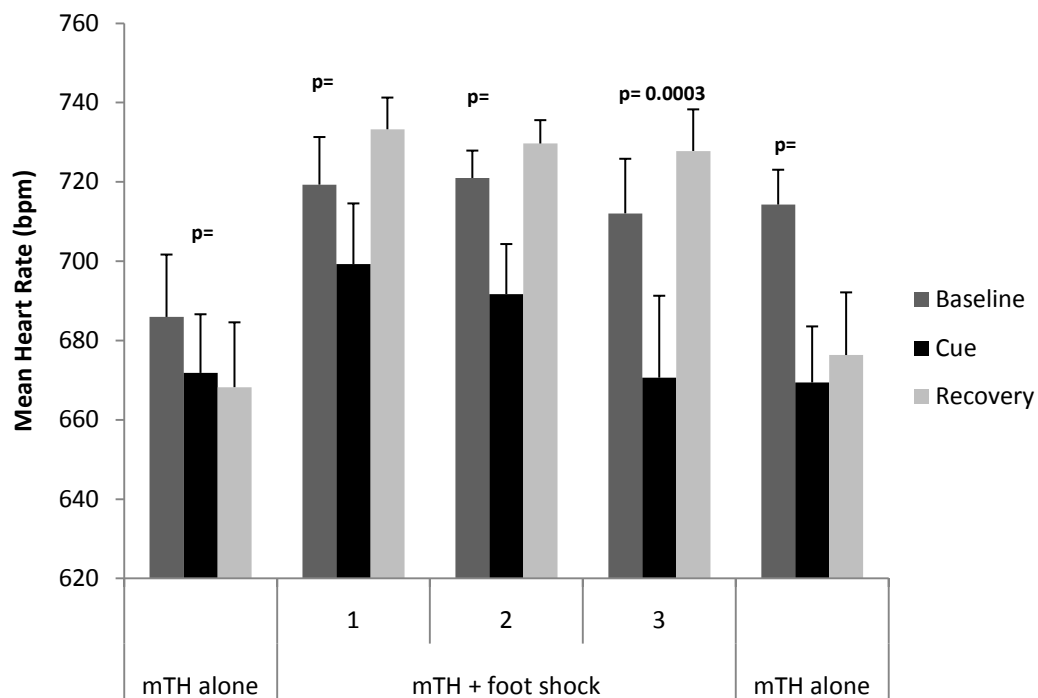


models have given new hope. What is known is that adequate sleep plays an important role in maintaining our physical and mental health and researchers must continue to combine existing treatments and implement new ones that hopefully in the future will identify the missing causal link between PTSD's nighttime and daytime symptoms.

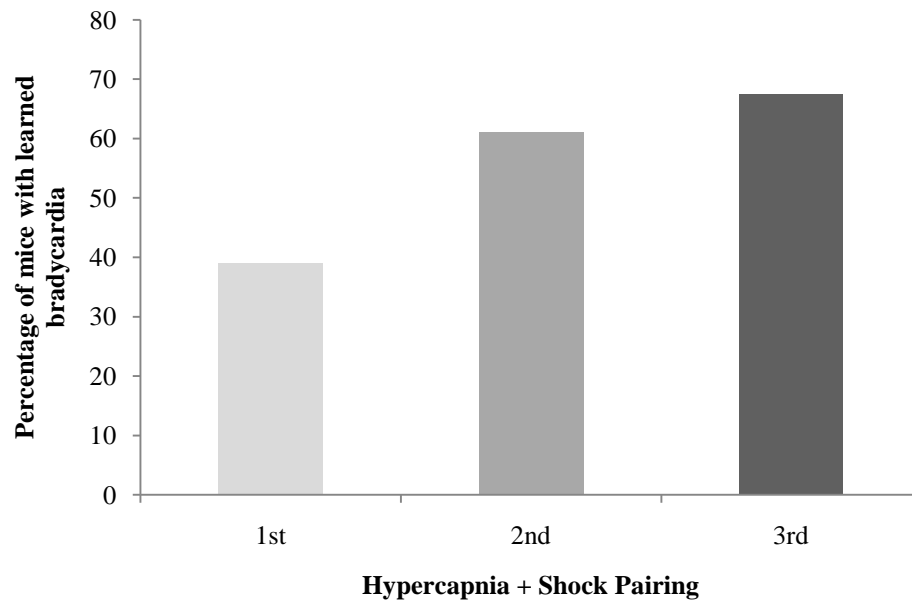
## APPENDIX A

### EXPERIMENT 1 DATA

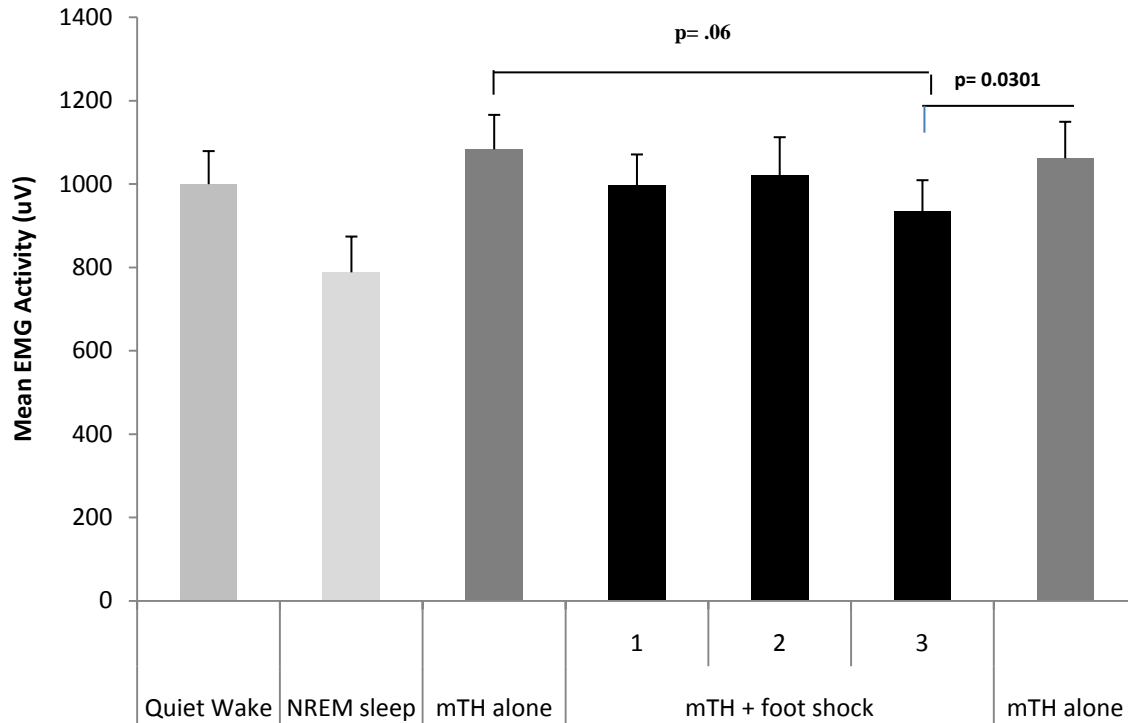
Data from Experiment 1 using mTH and tone are shown below



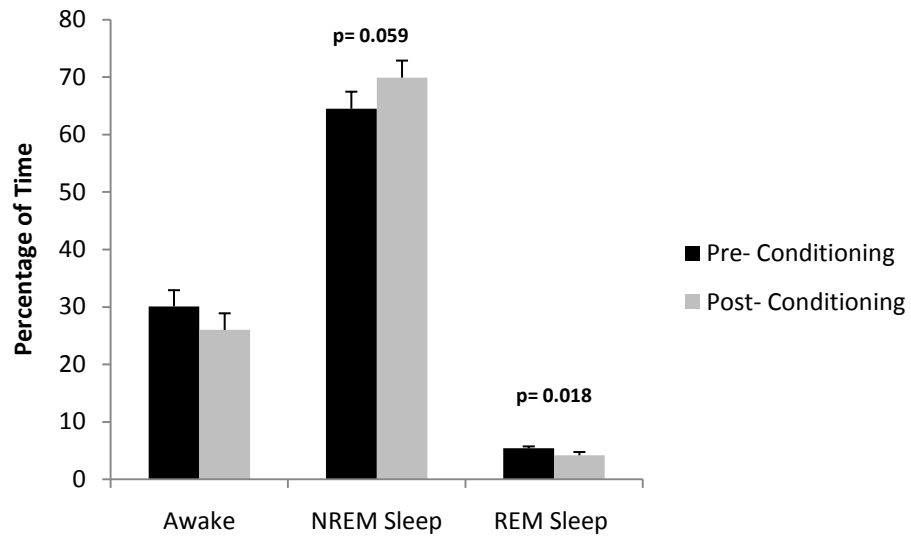
**Figure 4.1: Absolute heart rate (bpm) over type of stimulus presentation for baseline, cue and recovery time periods. Baseline refers to the 10 seconds directly before CS onset, cue period is the final 10 seconds of the ongoing CS directly before shock and recovery is the 10 seconds immediately after shock termination. Error bars represent the positive standard errors from the mean for each data point. P- values were calculated using paired t-tests and represent comparisons between baseline, cue and recovery periods.**



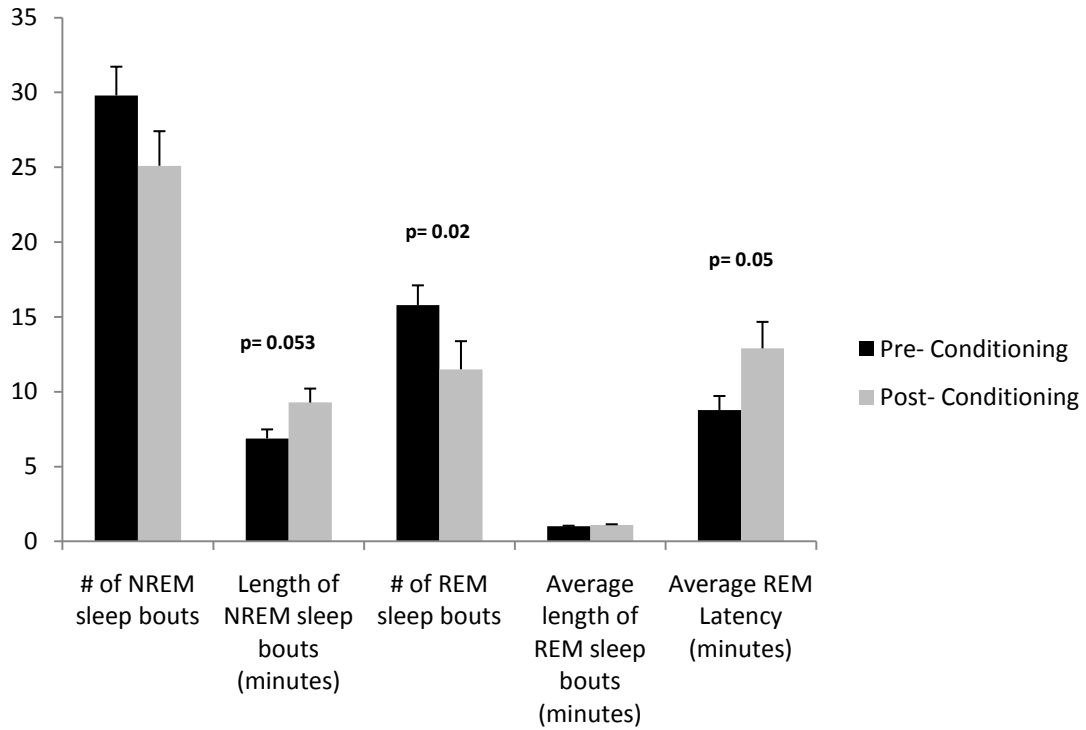
**Figure 4.2: Percentage of mice that developed and maintained learned bradycardic response from the 1<sup>st</sup> hypercapnia/ shock pairing to the 3<sup>rd</sup> (when compared to initial TH alone).**



**Figure 4.3: Mean EMG activity (uV) over 7 different time periods of interest before, during, and after conditioning. Measurements were taken during the 60 second increase in CO<sub>2</sub>. Error bars refer to the positive standard errors of the mean. P- values were calculated using paired t-tests and represent comparisons between (1) pre- conditioning mTH alone and tone + foot shock pairing, and (2) between mTH + foot shock pairings and post- conditioning tone alone.**



**Figure 4.4: Comparison of sleep state between 5 hour pre and post conditioning measurements during Experiment 1. Error bars represent the positive standard errors from the mean. P- values were calculated using paired t-tests and represent a comparison between pre and post conditioning.**



**Figure 4.5: Comparison of REM and NREM sleep during the 5 hours directly after conditioning and 5 hours before conditioning. Error bars refer to the positive standard errors of the mean. P- values were calculated using paired t-tests and represent a comparison between pre and post conditioning.**

## REFERENCES

- Albucher, R., & Liberzon, I. (2002). Psychopharmacological treatment in PTSD: A critical review. *Journal of Psychiatric Research*, 36, 355- 367.
- American Psychiatric A. *Diagnostic and Statistical Manual of Mental Disorders* (DSM- IV- TR). Washington, DC: American Psychiatric Association; 2000.
- Babson, K., Feldner, M., Trainor, C., & Smith, R. (2009). An experimental investigation of the effects of acute sleep deprivation on panic- relevant biological challenge responding. *Behavior Therapy*, 40(3), 239- 250.
- Benish, S., Imel, Z., & Wampold, B. (2008). Do all psychological treatments really work the same in posttraumatic stress disorder? *Clinical Psychology Review*, 30, 269- 276.
- Borbely, A., & Achermann, P. (1999). Sleep homeostasis and models of sleep regulation. *Journal of Biological Rhythms*, 14, 557–568.
- Breslau, N., Kessler, R., Chilcoat, H., Schultz, L., Davis, G., & Andreski, P. (1998). Trauma and posttraumatic stress disorder in the community. *Archives of General Psychiatry*, 55(7), 626- 632.
- Campan, M., Yugo, T., Jenkins, T., Balbir, A., & O' Donnell, C. (2005). Heart rate variability responses to hypoxic and hypercapnic exposures in different mouse strains. *Journal of Applied Physiology*, 99, 807- 813.
- Clum, G., Nishith, P., & Resick, P. (2001). Trauma- related sleep disturbances and self-reporting physical health symptoms in treatment- seeking female rape victims. *Journal of Nervous and Mental Disease*, 189(9), 618- 622.
- Forbes, D., Wolfgang, B., Cooper, J., Creamer, M., & Barton, D. (2009). Post- traumatic stress disorder- Best practice GP guidelines. *Australian Family Physician*, 38(3), 106- 111.
- Germain, A. (2009). Sleep disturbances in posttraumatic stress disorder. *Psychiatric Annals* 39(6), 335- 341.

- Germain, A., & O'Donnell, C. (2008). *A mouse model of sleep disturbances in ptsd: Concept presentation* [PowerPoint Slides]. Retrieve from University of Pittsburgh Multidisciplinary Sleep Conference, February 21, 2008.
- Germain, A., & O'Donnell, C. (2008). *Translational studies of fear- based sleep disturbances* [PowerPoint Slides]. Retrieve from University of Pittsburgh Multidisciplinary Sleep Conference, November 18, 2008.
- Green, B. (2003). Post- traumatic stress disorder: Symptom profiles in men and women. *Current Medical Research and Opinion*, 19(3), 200-204.
- Hamner, M., Lorberbaum, J., and George, M. (1999). Potential role of the anterior cingulate cortex in PTSD: Review and hypothesis. *Depression and Anxiety Journal*, 9, 1- 14.
- Harvey, A.G., & Bryant, R. (1998). The relationship between acute stress disorder and posttraumatic stress disorder: A prospective evaluation of motor vehicle accident survivors. *Journal of Consulting and Clinical Psychology*, 66, 507- 512.
- Harvey, A., Jones, C., and Schmidt, D.A. (2003). Sleep and posttraumatic stress disorder: A review. *Clinical Psychology Review*, 23(3), 377- 407.
- Hellman, K., and Abel, T. (2007). Fear conditioning increases NREM sleep. *Behavioral Neuroscience*, 121(2), 310- 323.
- Inman, D., Silver, S., Doghramji, K. (1990). Sleep disturbances in post- traumatic stress disorder: A comparison with non- PTSD insomnia. *Journal of Traumatic Stress*, 3, 429-437.
- Jha, S., Brennan, F., Pawlyk, A., Ross, R., & Morrison, A. (2005). REM sleep: A sensitive index of fear conditioning in rats. *European Journal of Neuroscience*, 21(4), 1077- 1080.
- Jukie, V., Sumie, J., Brecie, P., et al. (1999). Sleep disturbances and nightmares as symptoms of posttraumatic stress disorder. *Psychiatria Danubina*, 11, 13-17.
- Kobayashi, I., Boarts, J., and Delahanty, D. (2007). Polysomnographically measured sleep abnormalities in PTSD: A meta- analytic review. *Psychophysiology*, 44, 660- 669.
- Krakow, B., Artar, A., Warner, T., Melendrez, D., Johnston, L., Hollifield, M., Germain, A., & Koss, M. (2000). Sleep disorder, depression, and suicidality in female sexual assault survivors. *Crisis*, 21(4), 163- 170.
- Krakow, B., Germain, A., & Tandberg D., et al. (2000). Sleep breathing and sleep movement disorders masquerading as insomnia in sexual-assault survivors. *Comprehensive Psychiatry*, 41(1), 49-56.
- Kryger, M.H., Roth, T., & Dement, W.C. (2000): *Principles and practice of sleep medicine*, 3<sup>rd</sup> ed. Philadelphia: Saunders.



- Lamarche, L.J., & Koninck, J.D. (2007). Sleep disturbances in adults with posttraumatic stress disorder: A review. *Journal of Clinical Psychiatry*, 68, 1257- 1270.
- Lancee, J., Spoormaker, V.I., Krakow, B., and Bout, J. (2008). A systematic review of cognitive behavioral treatment for nightmares: Toward a well established treatment. *Journal of Clinical Sleep Medicine*, 4(5), 475-480.
- Lavie, P. (2001). Sleep disturbance in the wake of traumatic events. *New England Journal of Medicine*, 345, 1825- 1832.
- MacLean, R. (2007). The relationship between anxiety and sleep wake behavior after stressor exposure in the rat. *Brain Research*, 1164(20), 72-80.
- Manocchia, M., Keller, S., & Ware, J. (2001). Sleep problems, health- related quality of life, work functioning and health care utilization among the chronically ill. *Quality of Life Research*, 10(4), 331- 345.
- Mellman, T.A., Bustamante V., Fins A.I., Pigeon W.R., & Nolan B. (2002). REM sleep and the early development of posttraumatic stress disorder. *American Journal of Psychiatry*, 159(10), 1696-1701.
- Mellman, T., Pigeon, W., Nowell, P., & Nolan, B. (2007). Relationships between REM sleep findings and PTSD symptoms during the early aftermath of trauma. *Journal of Traumatic Stress*, 20(5), 893- 901.
- Mellman, T.A., Nolan, B., Hebding, J., Kulick- Bell, R., & Dominguez, R. (1977). A polysomnographic comparison of veterans with combat-related PTSD, depressed men, and non-ill controls. *Journal Sleep* 20(1), 46-51.
- Milad, M., Rauch, S., Pitman, R., Quirk, G. (2006). Fear extinction in rats: Implications for human brain imaging and anxiety disorders. *Biological Psychology*, 73, 61- 71.
- Neylan, T.C., Marmer, C.R., & Metzlet, T.J., et al. (1998). Sleep disturbances in the vietnam generation: Findings from a nationally representative sample of male Vietnam veterans. *American Journal of Psychiatry*, 155(7), 929- 933.
- Pace- Schott, E.F., Milad, M.R., Orr, S.P., Rauch, S.L., Stickgold, R., & Pitman, R.K. (2009). Sleep promotes generalization of extinction of conditioned fear. *Journal Sleep*, 32(1), 19- 26.
- Pawlyk, A.C., Jha, S.K., Brennan, F.X., Morrison A.R., & Ross, R.J. (2005). A rodent model of sleep disturbances in posttraumatic stress disorder: The role of context after fear conditioning. *Biological Psychiatry*, 57, 268- 277.

- Phillips, R., & LeDoux, J. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience*, 106(2), 274- 285.
- Pitman, R., Sanders, K., Zusman, R., Healy, A., Cheema, F., Lasko, N., Cahill, L., & Orr, S. (2002). Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biological Psychiatry*, 51(2), 189- 192.
- Quirk, G.J., Garcia, R., & Gonzalez- Lima, F. (2006). Prefrontal mechanisms in extinction of fear conditioning. *Biological Psychiatry*, 60, 337- 343.
- Rosen, J. (2006). Translational research: Parallels of human and animal research in biological psychology. *Biological Psychology*, 73, 1- 2.
- Saladin, M., Brady, K., Dansky, B., & Kilpatrick, D. (1995). Understanding comorbidity between PTSD and substance use disorders: Two preliminary investigations. *Addictive Behaviors*, 20(5), 643- 655.
- Sanford, L., Fang, J., & Tang, X. (2003). Sleep after differing amounts of conditioned fear training in BALB/ cJ mice. *Behavioral Brain Research*, 147, 193- 202.
- Shin, L & Liberzon, I. (2010). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology*, 35(1), 169- 191.
- Silvestri, A. (2005). REM sleep deprivation affects extinction of cued but not contextual fear conditioning. *Physiology and Behavior*, 84, 343- 349.
- Steenland, H & Zhou, M. (2009). Neck electromyography is an effective measure of fear behavior. *Journal of Neuroscience Methods*, 177(2), 355- 360.
- Stein, D., Ipser, J., & McAnda, N. (2009). Pharmacotherapy of posttraumatic stress disorder: A review of meta- analysis and treatment guidelines. *CNS Spectrums*, 14, 25- 31.
- Strine, T., & Chapman, D. (2004). Associations of frequent sleep insufficiency with health-related quality of life and health behaviors. *Sleep Medicine*, 6, 23- 27.
- Swanson, L., Favorite, T., Horin, E., & Arnedt, J. (2009). A combined group treatment for nightmares and insomnia in combat veterans: A pilot study. *Journal of Traumatic Stress*, 22(6), 639- 642.
- Van Cauter, E & Spiegel, K. (1999). Sleep as a mediator of the relationship between socioeconomic status and health: A hypothesis. *Annals of the New York Academy of Science*, 896, 254- 261.
- van der Kolk, B., Blitz, R., Burr, W., Sherry, S., & Hartmann, E. (1984). Nightmares and trauma: A comparison of nightmares after combat with lifelong nightmares veterans. *American Journal of Psychiatry*, 141(2), 187-190.

- Weiss, D., Marmar, C., Schlenger, W., Fairbank, J., Jordan, B., Hough, R., & Kulka, R. (1992). The prevalence of lifetime and partial post-traumatic stress disorder in Vietnam theater veterans. *Journal of Traumatic Stress*, 5(3), 365- 376.
- Yehuda, R. & Antelman, S. (1993). Criteria for rationally evaluating animal models of posttraumatic stress disorder. *Biological Psychiatry*, 33(7), 479- 486.
- Youakim, J., Doghramji, K., & Schutte, S. (1998). Posttraumatic stress disorder and obstructive sleep apnea syndrome. *Psychosomatics*, 39, 168- 171.

